Glioblastoma multiforme: a perspective on recent findings in human cancer and mouse models

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Gliomas are the most frequently occurring primary malignancies in the central nervous system, and glioblastoma multiforme (GBM) is the most common and most aggressive of these tumors. Despite vigorous basic and clinical studies over past decades, the median survival of patients with this disease remains at about one year. Recent studies have suggested that GBMs contain a subpopulation of tumor cells that displays stem cell characteristics and could therefore be responsible for in vivo tumor growth. We will summarize the major oncogenic pathways abnormally regulated in gliomas, and review the recent findings from mouse models that our laboratory as well as others have developed for the study of GBM. The concept of cancer stem cells in GBM and their potential therapeutic importance will also be discussed. [BMB reports 2011; 44(3): 158-164]

INTRODUCTION

Gliomas are the most common primary malignancies in the central nervous system (CNS). They are a heterogeneous group of tumors that display some histologic similarities to glia, which include astrocytes and oligodendrocytes. The main types of gliomas are astrocytomas, oligodendrogliomas, mixed gliomas, and ependymomas. Astrocytomas account for the majority of these tumors (1). Using the World Health Organization (WHO) classification system, gliomas can be classified into four different grades (2). Grade I and II low-grade astrocytomas are slow-growing less aggressive tumors while grade III and IV high grade gliomas are malignant tumors, characterized by high proliferation rate (grade III) and the presence of necrotic tissue and/or angiogenic activity (grade IV). The most malignant form, glioblastoma multiforme (GBM, grade IV), is one of the most aggressive and lethal forms of cancer. Despite intense investigation of this disease over the past few decades, most patients with GBM die within approximately 15 months of diagnosis (3, 4). Standard treatment consists of surgical removal of the tumor, followed by concomitant chemotherapy and radiotherapy. Temozolomide, an oral alkylating agent, is currently the most commonly used chemotherapy treatment (5).

GBMs can present as one of two distinct subtypes. Primary or "de novo" GBMs arise without any prior clinical or histological evidence of a lower grade precursor lesion and more commonly affect older patients (mean age of 62 years). Secondary or "progressive" GBMs progress from a lower grade glioma and typically develop in younger patients (median age of 45 years). According to a recent survey, primary GBMs constitute the majority of GBMs (95%) compared with secondary forms (6).

Here we will review the recent progress in our understanding of GBM with a focus on the abnormal signaling pathways involved in GBM, as well as mouse models employing known glioma mutations. The concept of "cancer stem cells" in glioma development will also be discussed, including its possible therapeutic implications.

ONCOGENIC PATHWAYS IN MALIGNANT GLIOMAS

Consistent with their high malignant potential, gliomas exhibit a vast array of well-documented genetic changes that likely contribute to their phenotype. Classical genetic pathways involved in gliomagenesis include mutations in genes involved in apoptosis as well as growth factor receptor signaling (Fig. 1) (4, 7). This underscores the critical role of genes that regulate cell survival and proliferation in the acquisition of the malignant phenotype.

The retinoblastoma and p53 tumor suppressors are central regulators of cell cycle control and programmed cell death, and are frequently mutated in malignant gliomas. Loss of function of these genes is also seen via amplification of the cyclin-dependent kinases Cdk4 and Cdk6, as well as inactivation of its negative regulator p16INK4a (8). P53 is frequently mutated in the DNA-binding region in both low- and high-grade gliomas as well as secondary and primary glioblastomas. P53 loss of function also occurs by amplification of the ubiquitin ligase Mdm2 or Mdm4, or loss of function of p14ARF, which antagonizes Mdm2 (4, 8, 9).
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Fig. 1. Oncogenic Pathways in Malignant Gliomas. Schema represents some of the most important genetic alterations found in human gliomas. Growth factor signaling, particularly epidermal and platelet-derived growth factor receptors (EGFR, PDGFR), leads to activation of its downstream effectors, such as Ras and Akt. These are in turn normally regulated by the Nf1 and Pten tumor suppressors, respectively, which are frequently mutated in GBMs. P53, Ink4a and other cyclin-dependent kinase inhibitors are other known tumor suppressors that are also commonly affected. New genes that have been discovered in recent sequencing efforts include metabolic genes such as Idh1.

Persistent activation of receptor tyrosine kinases (RTKs) is another frequently observed feature of malignant gliomas. Epidermal growth factor receptor (EGFR) mutations include amplifications, point mutations and deletions, with the most common alteration being the variant III deletion of the extracellular domain (EGFR-vIII mutant) (9). Platelet-derived growth factor receptor (PDGFR) and its ligands PDGF-A and PDGF-B are also commonly over-expressed in some glioma cells, raising the possibility of autocrine or paracrine activation (4). Other RTK genes, such as ERBB2, another member of the EGF receptor family, and MET, which encodes the hepatocyte growth factor receptor, have also been found to be mutated in GBMs (9).

RTKs mediate cell growth and proliferation via downstream effectors such as Ras and phosphatidylinositol-3-kinase (PI3K). Activity of these proteins is tightly regulated, especially by the tumor suppressors Nf1 and Pten. The neurofibromatosis gene Nf1 encodes the protein neurofibromin, which contains a functional RasGAP domain, thereby negatively regulating Ras activity (10). Patients with germline mutations in Nf1, called neurofibromatosis type I, have increased susceptibility to gliomas (11). The PI3K pathway is another essential survival pathway for a variety of cancer cells. The tumor suppressor Pten (phosphatase and tensin homologue on chromosome 10) negatively regulates the PI3K pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) back to phosphatidylinositol-4,5-biphosphate (PIP2) (12). Mutations in Pten frequently involve the phosphatase domain, and mutations in PI3K typically involve the catalytic (p110α) and regulatory (p85α) domains (9). Individuals with germline mutations in Pten (Cowden disease) also have increased incidence of developing gliomas compared to the general population (13).

In recent years, the glioblastoma genome has been studied in greater detail using multi-center patient samples and more sophisticated sequencing techniques. In 2009, The Cancer Genome Atlas Network (TCGA) reported their extensive genomic and functional genomic study on GBM with 206 human GBM samples (14). Comprehensive studies on DNA copy number, gene expression, DNA methylation, and nucleotide sequence aberrations were performed and revealed that RTK/Ras/PI3K, p53, and Pten alterations were among the most common alterations found in GBM. It also reported the most frequently mutated genes in human glioblastoma, which included P53, Pten, EGFR, CDKN2A/2B and NF1 (9), thus validating NF1 as a bona fide human glioblastoma tumor suppressor gene. Genomic profiling has also revealed new mutations in a subset of human gliomas, such as the metabolic enzyme gene isocitrate dehydrogenase (IDH1) (15, 16). Studies suggest that mutant IDH1 loses its normal enzymatic activity in tumors while gaining a new pro-oncogenic activity, leading to the production of an onco-metabolite (16-18). Other studies characterizing the genomic make-up of human glioblastoma have provided further insight into the genetic changes, core pathways and molecular subtypes underlying this disease (15, 19, 20).

Based on their gene expression profiles, TCGA further classified GMBs into four subtypes termed: Proneural, Neural, Classical, and Mesenchymal. While the status of gene expression and mutation in EGFR, NF1, PDGF, and IDH1 were defining components of these subtypes, it was also found that response to therapies was different for each subtype, suggesting that personalized treatment based on genomic alterations could lead to a more favorable outcome for this disease (21). This huge amount of genetic data from TCGA will allow a greater number of glioma-associated genes to be identified and subsequently validated in animal models. These models are powerful systems for understanding glioma biology and for use in pre-clinical testing (7).

MOUSE MODELS FOR GLIOMA

As discussed above, several mouse models have been devel-
oped and used for glioma studies. These models use different oncogenes and tumor suppressor genes to initiate tumors, as well as different systems to generate these mutations, including viral-mediated methods and Cre recombinase transgenesis. For example, Holland and colleagues used the RCAS-TVA system to activate Ras and Akt in the mouse brain (22). Replication-competent ALV splice-acceptor (RCAS) viral vectors harboring oncogenic Ras or activated Akt were developed, and the viruses that require TVA receptor for entry into cells were prepared. Both viruses were injected into the neonatal brain parenchyma where TVA expression was under the control of either the Nestin promoter or the GFAP promoter. They concluded from their studies that early progenitors or stem cells might be more tumorigenic, although the precise identification of the cells that produced the tumors was not part of that study (22). Marumato and colleagues developed lentiviral vectors encoding oncogenic Ras or activated Akt, with expression controlled by Cre recombinase. Injection of both viruses into hippocampus or subventricular zone (SVZ) of GFAP-Cre mice generated about 60 infected cells, and was sufficient to cause GBM development. Tumors were rarely detected when the lentiviruses were injected into cortex (23). These results are again consistent with the notion that the neural stem/progenitor cells are the cells of origin. Jacques and colleagues used GFAP-Cre adenovirus to inactivate Rb, p53, and/or Pten in GFAP-positive cells in SVZ and found that the injected mice developed glioma (24). Finally, orthotopic transplantation of neural stem cells or astrocytes with various genetic mutations into immunodeficient mice has also been used to investigate the roles of specific genes during glioma development. Bachoo and colleagues found that constitutively activated EGFR in an Ink4a/Arf−/− background is sufficient to induce high-grade gliomas in a transplantation model (25). BMI1 was also found to control glioma development in an Ink4a/Arf-independent manner in a transplantation model (26). Our group has generated unique mouse models of GBM using the Cre-loxP conditional knockout system. One allele of the Nf1 and p53 tumor suppressors is deleted through Cre-mediated recombination and germline mutation, respectively. This mouse (GFAP-Cre;Nf1−/−;p53−/−), referred to as Mut3 hereafter, exhibits no symptoms at birth; however, malignant astrocytomas develop with 100% penetrance (27). Consistent with studies on secondary human glioma tumor samples, the Mut3 tumors develop as low-grade infiltrative tumors (Grade II) that progress histopathologically through an anaplastic (Grade III) phase, and finally become GBM (Grade IV) tumors. The molecular characteristics of the Mut3 tumors also develop according to patterns described for the human counterpart tumors (27). Furthermore, we found that additional mutation of Pten (Mut6 mice, GFAP-Cre;Nf1−/−;p53−/−;Pten−/−) induces earlier onset of high-grade tumors that closely resemble primary GBM (28). This is consistent with the fact that Pten mutations are frequently observed in human primary GBM (29) and that decreased Pten expression is a marker of poor prognosis for patients with malignant glioma (19). As discussed above, Nf1, p53, and Pten are three of the most frequently mutated tumor suppressor genes in human GBMs according to a recent study from TGCA (14); thus, our mouse models offer a physiologically relevant system for studying the initiation and progression of these tumors.

A subsequent generation of glioma mouse models specifically targeted the neural stem/progenitor cells using a Nestin-CreER driver. CreER is a fusion protein consisting of Cre recombinase linked to a modified estrogen receptor ligand-binding domain and can mediate loxP recombination when CreER is translocated into the nucleus upon treatment of the mice with tamoxifen (30). Mice with tumor suppressor inactivation (Nestin-CreER;Nf1−/−;p53−/−;Pten−/−) induced by tamoxifen developed malignant glioma supporting the notion that mutation of these tumor suppressors in the SVZ stem/progenitor cells is sufficient to give rise to glioma. The use of adenov-cre stereotactic injection experiments in which cre-virus was either injected into the SVZ of three strains of tumor suppressor floxed mice (Nf1−/−p53−/−, Nf1−/−p53−/−, and Nf1−/−p53−/−;Pten−/−) or in other regions of the parenchyma further demonstrated the inability of non-stem/progenitor cells to give rise to gliomas. While tumor suppressor inactivation in the SVZ at both early postnatal and adult ages induced tumors with 100% penetrance, we rarely observed tumor formation when the viruses were injected into non-neurogenic regions, such as cortex and striatum (31). Together, these data provide strong evidence that the cells of tumor origin in our mouse GBM models are the neural stem/progenitor cells.

Mouse models are powerful tools to study tumor physiology and the molecular mechanisms underlying the initiation and progression of cancer. For example, studies with pre-symptomatic mice permit researchers to investigate when and where tumors begin to develop, which is not possible in patient studies. From our studies with the GFAP-cre conditional knockout mice (Mut3 and Mut6), the earliest abnormal event observed in pre-symptomatic mice was the abnormal migration of mutant cells away from the SVZ region, again supporting the notion that the SVZ stem/progenitor cells are the cell of origin in glioma (27, 28). Our recent studies with the Nestin-creER mice and the cre virus stereotactic injection experiments, together with the studies from other groups, strongly support this model (31). Identification of this cell population as cells that can give rise to tumors should provide new insights into glioma development and offer novel strategies for the treatment of this devastating disease.

STEM CELLS AND GLIOMA STEM CELLS

Stem cells are cells that maintain both the capacity for self-renewal and the potential to differentiate into mature cells. In general, only a small subset of cells in tissues has these properties. To date, a number of organs have been shown to contain stem cells, including blood, brain, prostate, colon, lung,
pancreas, skin, and mammary gland (32-40). Isolation of stem cells is largely dependent upon cell surface marker selection and/or special culture conditions that specifically allow for the growth of stem/progenitor cells while diluting out differentiated cells. For example, hematopoietic stem cells (HSCs) can be sorted by expression of Lin (negative), Sca-1 (positive), c-kit (positive), CD34 (negative), and Flk2 (negative) markers. It is believed that a small population, less than 0.1%, of mouse bone-marrow cells has the capacity for self-renewal and multipotency, and these are comprised of long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors (41-43). In the prostate, adult stem cells were found in the region proximal to the urethra (44). These cells are capable of self-renewing, differentiating, and regenerating tissue (45). Prostate stem cells can be sorted by CD49f/Sca-1 surface markers, and Bmi1 has been shown to play a critical role in the regulation of prostate stem cell self-renewal and malignant transformation (46). In the mammalian brain, neural stem cells are found in two specific locations: the subventricular zone (SVZ), adjacent to the lateral ventricles, and the sub-granular zone (SGZ) of the dentate gyrus in the hippocampus (34, 35). It had been generally believed that normal mammalian brain contains only terminally differentiated cells, neurons and astrocytes, and lacked mitotic activity. However, astrocytes in the SVZ are labeled with proliferation markers and give rise to new neurons in the olfactory bulb. When dividing cells are ablated by treatment with Ara-C, an anti-mitotic agent, SNZ astrocytes (Type B cells) can divide to produce immature precursors and neuroblasts (34). The dentate gyrus (DG) of the hippocampus is another neurogenic region in mammalian brain; neural stem cells produce immature precursors that give rise to new neurons that locally migrate within the granular cell layer of the DG (47, 48). Cells isolated from the DG can be cultured in vitro in defined medium containing basic fibroblast growth factor, passaged, and induced to differentiate into neurons and glial cells (35).

For certain types of cancers, it has been proposed that these multipotent cells in normal tissues are the origin of the cancer. This is supported by the observation that cancer cells share more similarities with stem cells than differentiated cells, so fewer mutations are required for malignant transformation. In addition, the lifetime of stem cells is much longer than mature cells, allowing stem cells greater opportunity to acquire mutations. As normal stem cells can coordinate development of organs, cancer can be considered as abnormal tissue initiated by a cell in which critical mutations have occurred. Although normal stem/progenitor cells are likely to be the cell of origin in diverse cancers, it is worthy to note that "cancer stem cells" do not necessarily originate from normal multipotent cells. Rather, unlike other cells of the tumor, they can self-renew and differentiate, and are sufficient to generate a tumor. The concept of "cancer stem cells" came from studies on hematopoietic stem cells and leukemia (49). Just as normal HSCs can self-renew and differentiate into mature cells, constituting the whole hematopoietic system, cancer stem cells can initiate and maintain the tumor. Using cancer stem cells isolated from patients with acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), or chronic myelogenous leukemia (CML), Dick and colleagues showed these cells could engraft and proliferate in immunodeficient mice, recapitulating human leukemia in recipient mice (50-52).

Involvement of cancer stem cells in the pathogenesis of brain tumors was reported by Dirks and colleagues, who isolated cells from primary human brain tumors using selection in neurosphere culture (53, 54). The neurosphere method was originally developed for the in vitro culture of neural stem and progenitor cells, whereby progenitor cells are grown in serum-free conditions and supplemented with growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (55, 56). When tumor cells from medulloblastoma and GBMs were passaged in neurosphere culture conditions and then transplanted into immunodeficient mice, these "cancer stem cells" were shown to induce tumor formation (53, 54). These "cancer stem cells" were later identified

![Fig. 2. Glioma stem cells in GBM treatment. (a) Model for conventional therapy with temozolomide and radiation. The tumor regresses after treatment, however it recurs because the surviving glioma stem cells can regenerate the tumor. (b) Model for the glioma stem cell-targeting therapy. Tumors regress gradually because only the differentiated tumor cells survive.](http://bmbreports.org)
using the CD133 marker, which greatly enriches the population of tumor-propagating cells compared to the CD133 population. These were found to be highly resistant to chemotherapy and radiotherapies (57, 58), offering a possible explanation for the failure of current GBM treatments (Fig. 2). Following irradiation, either in vitro or in vivo, the population of tumor cells with stem cell properties increased over time due to up-regulation of the DNA damage checkpoint response to radiation (59). Induction of cell differentiation with bone morphogenetic protein (BMP), which is involved in the differentiation of neural stem cells during development, was sufficient to inhibit tumor development in vivo, suggesting that the stem cell properties of these cells are required for their tumorigenicity (60).

As discussed earlier, we have shown that neural stem/progenitor cells are the tumor-propagating cells in our mouse models of malignant astrocytoma. Similar to the findings of Singh et al., stem-like cancer cells can be isolated from the tumors that develop in our glioma mouse models. These glioma stem cells can be propagated as self-renewing cultures that exhibit abnormal growth properties and, when cultured under differentiation conditions, are able to express markers of the three major neural lineages, indicating a stem-cell like property (28, 31). Further demonstrations of the in vitro and in vivo properties of glioma stem cells are under way and will be essential in determining their role in glioma progression. These studies will be vital in finding ways to overcome the therapeutic resistance of these highly malignant cancers.

CONCLUSION

Although previous research on GBM has not yet translated into significant advances in GBM treatment, it has clearly extended our understanding of the nature of this disease. We now know many of the critical pathways involved in GBM tumor initiation and progression, and extensive clinical and molecular data from hundreds of patients have been acquired. The identification of a subpopulation of GBM tumor cells with stem cell-like properties provides new insights into GBM tumorigenesis and offers novel targets for the treatment of this disease. An intriguing and important question remains: whether targeting the cancer stem cells can improve treatment success for GBM (Fig. 2). If so, new strategies that effectively target the chemotherapeutic and radio-resistant cancer stem cells may greatly improve the prognosis of this disease, which is incurable to date.

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